Enhancement of Rostral Ventrolateral Medulla Neuronal Nitric-Oxide Synthase–Nitric-Oxide Signaling Mediates the Central Cannabinoid Receptor 1-Evoked Pressor Response in Conscious Rats

Badr Mostafa Ibrahim and Abdel A. Abdel-Rahman

Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, North Carolina

Received January 24, 2012; accepted February 23, 2012

ABSTRACT

Our recent studies implicated brainstem GABAergic signaling in the central cannabinoid receptor 1 (CB₁R)-mediated pressor response in conscious rats. Given the well established link between neuronal nitric-oxide synthase (nNOS)/nitric oxide (NO) signaling and GABAergic transmission in brainstem cardiovascular regulating areas, we elucidated the role of nNOS-generated NO in the central CB₁R-elicited pressor response. Compared with vehicle, intracisternal (i.c.) microinjection of the CB₁R agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate (WIN55212-2) (15 μ g/rat) significantly enhanced nNOS phosphorylation as well as the total nitrate and nitrite content in the rostral ventrolateral medulla (RVLM) at 5, 10, and 30 min, which paralleled the elicited pressor response. These findings were corroborated by: 1) the parallel dose-related increases in blood pressure and RVLM-NO levels, measured in real

time by in vivo electrochemistry, elicited by intra-RVLM WIN55212-2 (100, 200, or 300 pmol /80 nl; *n* = 5) in conscious rats; and 2) the significantly higher phosphorylated nNOS (p-nNOS) levels in the WIN55212-2-injected RVLM compared with the contralateral RVLM. Subsequent neurochemical studies showed that WIN55212-2 (15 μ g/rat i.c.) significantly increased the number and percentage of neurons immunostained for nNOS (nitroxidergic neurons) and c-Fos (marker of neuronal activity) within the RVLM. The increases in blood pressure and the neurochemical responses elicited by intracisternal WIN55212-2 were attenuated by prior central CB₁R blockade by *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251; 30 μ g/rat i.c.) or selective nNOS inhibition by *N*^ω-propyl-L-arginine (1 μ g/rat i.c.). These findings implicate RVLM p-nNOS/NO signaling as a molecular mechanism in the central CB₁R-evoked pressor effect in conscious rats.

Introduction

Enhanced phosphorylation of nNOS, which leads to NO release, underlies a wide array of CB₁R-mediated neuropharmacological actions (Azad et al., 2001; Makara et al., 2007; Jones et al., 2008; Carney et al., 2009). For example, CB₁R activation in CA1 hippocampal pyramidal neurons leads to nNOS-NO-dependent suppression of GABAergic neurotrans-

mission (Makara et al., 2007); further, nNOS-derived NO in the brainstem is involved in central blood pressure regulation (Martins-Pinge et al., 1999, 2007; Mayorov, 2007; Nassar and Abdel-Rahman, 2008). It is noteworthy that our previous studies implicated the inhibition of GABAergic neurotransmission in the central CB₁R-mediated sympathoexcitation/pressor response in conscious rats (Ibrahim and Abdel-Rahman, 2011), and reduced GABA release in the RVLM, caused by nNOS-derived NO, was associated with a pressor response (Chan et al., 2003; Martins-Pinge et al., 2007).

One possible signaling pathway that might underlie CB₁R-mediated nNOS activation is ERK1/2 phosphorylation. This possibility gains support from our recent finding that ERK1/2 phosphorylation in the RVLM contributed to the CB₁R-medi-

This work was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant 2R01-AA07839-18] and in part by the El-Minia Faculty of Pharmacy via a scholarship provided by the Egyptian Government (Scholarship Missions Program, Ministry of Higher Education) (to B.M.I.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
<http://dx.doi.org/10.1124/jpet.112.192369>.

ABBREVIATIONS: nNOS, neuronal nitric-oxide synthase; p-nNOS, phosphorylated nNOS; t-nNOS, total nNOS; NO, nitric oxide; NOx, total nitrate/nitrite; nNOS-ir, nitroxidergic immunoreactive neurons; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; ANOVA, analysis of variance; BP, blood pressure; CB₁R, cannabinoid receptor 1; ERK1/2, extracellular signal-regulated kinase 1/2; DMSO, dimethyl sulfoxide; HR, heart rate; i.c., intracisternal; MAP, mean arterial pressure; NPLA, *N*^ω-propyl-L-arginine; RVLM, rostral ventrolateral medulla; WIN55212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl) methanone mesylate salt.

ated pressor response in conscious rats (Ibrahim and Abdel-Rahman, 2012). Furthermore, ERK1/2-dependent phosphorylation of nNOS in the RVLM is implicated in sympathoexcitation (Chan et al., 2004, 2005, 2010). Together, these findings highlight a potentially important, yet unexplored, role for nNOS/NO signaling in the RVLM in the pressor response elicited by central CB₁R activation.

The aim of the present study was to test the hypothesis that brainstem nNOS/NO signaling contributes to the central CB₁R-mediated pressor response. Because the pressor effect of (*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt (WIN55212-2) in our previous studies (Ibrahim and Abdel-Rahman, 2011, 2012) peaked at approximately 10 min and subsided by 30 min, we investigated the effect of intracisternal WIN55212-2 on nNOS phosphorylation and total nitrate/nitrite (NO_x) in the RVLM at 5, 10, and 30 min after WIN55212-2. It was also important to determine whether the dose-related increases in BP elicited by intracisternal WIN55212-2 in our recent studies (Ibrahim and Abdel-Rahman, 2011, 2012) were replicated by local activation of RVLM CB₁R and to establish a temporal relationship between locally generated NO and the pressor response. To achieve this goal, we measured concomitant real-time changes in RVLM NO (in vivo electrochemistry) and BP before and after intra-RVLM WIN55212-2 or its vehicle; the electrochemical (NO level) findings were confirmed at the conclusion of the study by comparing the level of p-nNOS in the microinjected versus the contralateral RVLM. Finally, to ascertain causal involvement of nNOS/NO signaling in the central CB₁R-mediated pressor response, we investigated the impact of prior selective inhibition of central nNOS with *N*^ω-propyl-L-arginine (NPLA) on central CB₁R-mediated pressor response and the associated increases in nNOS phosphorylation level as well as the number of neurons that exhibited dual immunostaining for nNOS [nitroxidergic immunoreactive neurons (nNOS-ir)] and c-Fos (marker of neuronal activity) within the RVLM. The integrative studies were conducted in conscious unrestrained rats, and the brains were collected at the conclusion of cardiovascular measurements for the molecular studies.

Materials and Methods

Male Sprague-Dawley rats (300–350 g; Charles River, Raleigh, NC) were housed two per cage in a room with controlled environment at a constant temperature of 23 ± 1°C, humidity of 50 ± 10%, and a 12-h light/dark cycle. Food (Prolab Rodent Chow; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical, experimental, and animal care procedures were performed in accordance with and approved by the East Carolina University Institutional Animal Care and Use Committee and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory and Animal Resources, 2010).

Intra-Arterial Catheterization, Intracisternal, and Intra-RVLM Cannulation. Under sterile conditions and anesthesia, ketamine (9 mg/100 g) and xylazine (1 mg/100 g i.p.), an arterial catheter for BP measurement was placed into the abdominal aorta via the femoral artery, and a guide cannula for intracisternal injections was implanted into the cisterna magna as detailed in our previous study (Ibrahim and Abdel-Rahman, 2012). For intra-RVLM cannulation, the method described in our previous studies was followed (Li et al., 2005; Zhang and Abdel-Rahman, 2005; Li and Abdel-Rahman, 2007). A stainless-steel guide cannula (21.5 gauge; 14 mm in length)

was implanted 2 mm above the RVLM level at coordinates of −2.8 posterior, ± 2 lateral, and −0.5 mm dorsoventral with the interaural line as the reference according to Paxinos and Watson (2005).

Blood Pressure and Heart Rate Measurements. Procedures detailed in our previous studies were used for BP and HR measurements in conscious unrestrained rats (Ibrahim and Abdel-Rahman, 2011, 2012; Nassar et al., 2011).

Real-Time Measurement of RVLM NO and Drug Microinjections. Procedures of preparation and calibration of the carbon fiber electrodes for detection of changes in basal NO levels in the RVLM have been detailed previously (Friedemann et al., 1996; Li and Abdel-Rahman, 2009). A probe that combines a stainless-steel injector (30 gauge; 21.5 mm in length) and the NO-carbon fiber electrode to permit intra-RVLM microinjections was inserted directly into the RVLM of unrestrained rats via the preimplanted guide cannula. Real-time changes in RVLM NO were measured with the IVEC-10 system (Medical Systems Corp., Greenvale, NY), which has a detection limit of 35 ± 7 nM NO (Friedemann et al., 1996). The injector was connected to PE-10 tubing via PE-50 tubing attached to a Hamilton microsyringe (1 µl) (Hamilton Co., Reno, NV). Identification of the RVLM was based on obtaining abrupt elevation in mean arterial pressure (MAP) (≥ 33 mm Hg) and bradycardia (≥ −65 beats/min) in response to L-glutamate (1 nmol) microinjecting at the beginning of the experiment and by histological verification at the end of the experiment after fast green microinjection (40 nl) as detailed previously (Li et al., 2005). Animals that failed the positive verification were excluded from the study.

Total Nitrate and Nitrite Measurement. Protocol from our previous studies was followed for measurement of NO_x as an index for NO levels in the RVLM (Bender and Abdel-Rahman, 2010).

Western Blot Analysis. A modified protocol from our recent studies (Nassar et al., 2011; Ibrahim and Abdel-Rahman, 2012) was used to measure phosphorylated and total nNOS. Animals were euthanized, and brains were removed, flash-frozen, and stored at −80°C until used. Tissues were collected from RVLM at −12.8 to −11.8 mm relative to bregma (Paxinos and Watson, 2005) with a 0.75-mm punch instrument (Stoelting Co., Wood Dale, IL). Equal amounts of protein from each sample were separated by gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and then incubated overnight at 4°C with a mixture of rabbit antiphospho-nNOS (Ser1417) antibody (1:500; Thermo Fisher Scientific, Waltham, MA) and mouse polyclonal anti-nNOS antibody (1:500; BD Biosciences, San Jose, CA). Membranes were washed four times with phosphate-buffered saline containing 0.1% Tween 20 then incubated for 60 min with mixture containing IRDye680-conjugated goat anti-mouse and IRDye800-conjugated goat anti-rabbit (1:5000; LI-COR Biosciences). Bands representing phosphorylated and total protein were detected simultaneously by using Odyssey Infrared Imager and analyzed with Odyssey application software version .3 (LI-COR Biosciences). All data were averaged values of integrated density ratio of p-nNOS normalized to the corresponding total nNOS (t-nNOS) and expressed as percentage of control (vehicle-treated rats). In experiment 2, changes in the RVLM p-nNOS/t-nNOS ratio were detected in the injected site (intra-RVLM) and compared with those of the contralateral site (control).

Immunofluorescence. Protocol used in previous reports was used for nNOS-ir and c-Fos-immunoreactive neurons colocalization studies (Ibrahim and Abdel-Rahman, 2011) in the RVLM, rostrally from −12.8 to −11.8 mm relative to bregma (Paxinos and Watson, 2005). Sections were incubated for 48 h at 4°C in a mixture of mouse anti-nNOS (1:200; BD Biosciences) and rabbit polyclonal anti-c-Fos antibody (1:2000; Calbiochem, San Diego, CA). Dual-labeling immunofluorescence was revealed by incubation for 2 h in a mixture of fluorescein isothiocyanate-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit (1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). A Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, New York) was used for the visualiza-

tion, acquisition, and quantification of colocalization. Four to six sections per animal at the level of RVLM were examined (Paxinos and Watson, 2005).

Drugs. WIN55212-2 and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO). *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) was purchased from Cayman Chemical (Ann Arbor, MI). The emulsifier Alkamuls EL620 (polyethoxylated castor oil) was purchased from Rhodia (Cranbury, NJ). WIN55212-2 and AM251 were dissolved in a mixture (1:1:18) of dimethyl sulfoxide/Alkamuls/sterile saline. NPLA was purchased from Tocris Biosciences (Ellisville, MO) and dissolved in sterile saline (Bender and Abdel-Rahman, 2010).

Protocols and Experimental Groups

Experiment 1: Effect of Intracisternal WIN55212-2 on RVLM nNOS Phosphorylation and NO_x Content. To determine whether RVLM-nNOS/NO signaling is implicated in central CB₁R-mediated pressor response, we investigated the effect of intracisternal WIN55212-2 on nNOS phosphorylation and NO_x content in the RVLM. Brain tissues were collected from animals sacrificed after vehicle (control) or 5, 10, and 30 min after WIN55212-2 (15 µg i.c.) ($n = 4-6$ each) and prepared for Western blotting to detect changes in p-nNOS and NO_x content in the RVLM as detailed under *Materials and Methods*. The chosen dose of WIN55212-2 (15 µg i.c.) and time points were based on our recent studies (Ibrahim and Abdel-Rahman, 2011, 2012).

Experiment 2: Effects of Intra-RVLM Microinjection of WIN55212-2 on BP and RVLM p-nNOS/NO. In this experiment we used a technique (Li and Abdel-Rahman, 2009) that permitted simultaneous measurements of BP, HR, and real-time changes in NO in the RVLM of conscious rats ($n = 5$) that received 80 nl of vehicle (control) followed by three successive doses of the CB₁R agonist WIN55212-2 (100, 200, and 300 pmol) at 10-min intervals; injections were made unilaterally into the RVLM; the 300-pmol WIN55212-2 dose is equivalent to 0.16 µg. It is noteworthy that the use of previously reported dose ranges (0.5–50 pmol) of WIN55212-2, which produced pressor response in anesthetized rats (Padley et al., 2003), failed to produce any changes in BP or basal RVLM NO in our hands. It is noteworthy that this finding, which might be caused by the use of conscious rats, indicated that the responses elicited by the higher doses of WIN55212-2 were caused by the drug and ruled out the potential contribution of volume or vehicle effect. Ten minutes after the last dose, the rats were euthanized, and a 0.75 micropunch instrument was used to collect two micropunches from the injected and contralateral RVLM as detailed under *Materials and Methods* for determining changes in nNOS phosphorylation (Western blotting). The contralateral (noninjected) RVLM served as control.

Experiment 3: Effect of Central nNOS Inhibition on the Pressor Response Elicited by Intracisternal WIN55212-2. To establish a causal role between nNOS activation, NO production in the RVLM, and central CB₁R-mediated pressor response, we investigated the effect of central nNOS inhibition on WIN55212-2-mediated pressor response in conscious rats ($n = 5-8$) that received the selective nNOS inhibitor NPLA (1 µg i.c.), or equal volume of sterile saline, 30 min before WIN55212-2 (15 µg i.c.) or an equal volume of its vehicle. The selected NPLA dose was used

in our previous studies (Bender and Abdel-Rahman, 2010). Cardiovascular measurements were continued for 30 min after WIN55212-2 or the vehicle. At the conclusion of the integrative studies, we investigated the changes in RVLM p-nNOS levels (Western blotting) caused by WIN55212-2 in the absence or presence of CB₁R blockade (AM251; 30 µg i.c.) or nNOS inhibition (NPLA; 1 µg i.c.). The neurochemical studies on AM251 were conducted in the brains of animals used in our recent studies, which demonstrated AM251 abrogation of WIN55212-2 evoked pressor response in the same animal model (Ibrahim and Abdel-Rahman, 2011, 2012). In additional groups of animal treated as above ($n = 4-5$ each) brain tissues were collected after transcardiac perfusion, and the number of neurons that exhibited dual immunostaining for nNOS (nNOS-ir) and c-Fos (marker of neuronal activity) in the RVLM by dual immunofluorescence was quantified.

Statistical Analysis

Mean arterial pressure and heart rate (HR) are expressed as mean $\pm$ S.E.M. change from their respective baseline values after the pretreatment and before WIN55212-2 or vehicle injection. Data were then analyzed by repeated-measures ANOVA using the SPSS 16.0 statistical package for Windows (SPSS Inc., Chicago, IL) as detailed previously (Ibrahim and Abdel-Rahman, 2012). Western blot data for each protein were expressed as percentage of control (vehicle alone) value and analyzed by unpaired *t* test using Prism version 5 for Windows (GraphPad Software Inc., San Diego, CA). Furthermore, one-way ANOVA, followed by multiple comparison post hoc, was conducted to determine the effects of WIN55212-2 alone and in the presence of AM251 or NPLA on nNOS phosphorylation or dual nNOS-ir/c-Fos immunostaining. To achieve this goal, WIN55212-2 values were compared with those of the control (vehicle alone) and the corresponding values in animals pretreated with AM251 or NPLA. $P < 0.05$ was considered significant.

Results

Intracisternal WIN55212-2 Increased RVLM nNOS Phosphorylation and NO_x Levels. In this experiment, we investigated the changes in RVLM nNOS phosphorylation and NO_x content at 5, 10, and 30 min after WIN55212-2 (15 µg i.c.). Compared with the control group, sacrificed 5 min after intracisternal vehicle ($n = 4$), WIN55212-2 ($n = 6$) significantly ($P < 0.05$) increased p-nNOS as well as the NO_x in the RVLM at all measured time points in parallel to the elicited pressor response as shown in Fig. 1.

Intra-RVLM WIN55212-2 Increases BP and RVLM NO. These studies were undertaken to directly elucidate the role of RVLM nNOS/NO pathway in the pressor response elicited by local CB₁R activation. Whereas unilateral intra-RVLM microinjection of 80 nl of vehicle or lower doses of WIN55212-2 (see *Materials and Methods*) had no effect on BP, HR, or RVLM NO, microinjection of 100, 200, or 300 pmol WIN55212-2 dose-dependently ($P < 0.05$) increased MAP, HR, and RVLM NO levels (Figs. 2 and 3). In addition, p-nNOS levels were significantly ($P < 0.05$) elevated in the WIN55212-2-treated rats compared with the contralateral (control) RVLM (Fig. 3).

Fig. 1. Effect of intracisternal WIN55212-2 (15 $\mu\text{g}/\text{rat}$) on RVLM p-nNOS levels (A) and total nitrate/nitrite levels in the RVLM (NOx content; index of NO) (B). Brains were collected 5 min after vehicle or 5, 10, or 30 min after WIN55212-2. Data are presented as averaged values of integrated density ratio of p-nNOS to its total protein (t-nNOS) and expressed as the percentage of control (vehicle). NOx data are the mean $\pm$ S.E.M of four to six observations/group. *, $P < 0.05$ versus vehicle.

Selective Central Inhibition of nNOS Phosphorylation Abrogates Central WIN55212-2-Evoked Pressor Response. To investigate a causal role for brainstem RVLM nNOS phosphorylation in the central CB₁R-mediated pressor response, conscious rats were pretreated with the selective nNOS inhibitor NPLA (1 μg i.c.) before WIN55212-2 (15 μg i.c.). Baseline MAP and HR were similar in all groups of rats used in this study (Table 1). Furthermore, MAP and HR were similar after saline or NPLA at the time of WIN55212-2 or vehicle injection (Table 1). As shown in Fig. 4, pretreatment

with NPLA significantly ($P < 0.05$) attenuated the pressor, but not the bradycardic response, elicited by WIN55212-2.

Central CB₁R Blockade or nNOS Inhibition Abrogates WIN55212-2 Enhancement of RVLM nNOS Activation. As shown in Fig. 5, WIN55212-2 (15 μg i.c.; $n = 5$) significantly ($P < 0.05$) increased RVLM nNOS phosphorylation compared with control (vehicle). NPLA (1 μg i.c.; $n = 5$) significantly ($P < 0.05$) reduced, whereas AM251 (30 μg i.c.; $n = 4$) had no effect on, p-nNOS levels in the RVLM compared with control values ($n = 4$). Nonetheless, central CB₁R blockade (AM251; $n = 5$) or nNOS inhibition (NPLA; $n = 5$) fully abrogated WIN55212-2-evoked enhancement of p-nNOS levels when comparisons were made with the values of vehicle, AM251, or NPLA alone. Representative p-nNOS/t-nNOS bands from all groups are shown in Fig. 5.

In a parallel experiment, c-Fos expression was quantified in RVLM nNOS-ir neurons by dual labeling immunofluorescence. WIN55212-2 (15 μg i.c.) significantly ($P < 0.05$) increased the percentage of nNOS-ir neurons expressing c-Fos compared with control (Fig. 6). NPLA (1 μg i.c.) or AM251 (30 μg i.c.) did not significantly alter the basal nNOS-ir/c-Fos ratio. It is noteworthy that pharmacological intervention with AM251 or NPLA significantly ($P < 0.05$) attenuated WIN55212-2-evoked increases in the number and percentage of RVLM neurons that exhibited dual immunostaining for nNOS and c-Fos. As shown in Fig. 6, the total number of nNOS-ir neurons in the RVLM was similar in all groups.

Discussion

In this study, we tested the hypothesis that RVLM nNOS/NO signaling is pivotal for the central CB₁R-mediated pressor response. The most important findings of this study are: 1) intracisternal WIN55212-2-evoked pressor response in conscious rats is associated with significant enhancement in RVLM nNOS phosphorylation and NOx content; 2) localized CB₁R activation in the RVLM dose-dependently increased MAP, HR, and NO (in vivo electrochemistry) and enhanced nNOS phosphorylation; 3) WIN55212-2-evoked neurochemical responses were attenuated by central CB₁R blockade (AM251); and 4) prior inhibition of central nNOS (NPLA) abrogated the WIN55212-2-evoked increases in blood pressure, nNOS phosphorylation, and number of activated nNOS-ir neurons (c-Fos) in the RVLM. Collectively, these findings identify the enhancement of RVLM nNOS/NO signaling as a molecular mechanism for the central CB₁R-mediated pressor response in conscious rats.

We hypothesized a crucial role for RVLM nNOS-derived NO in the central CB₁R-mediated pressor response in con-

Fig. 2. Typical tracings depicting the dose-dependent real-time changes in RVLM-NO (detected electrochemically in RVLM by a nafion-coated microelectrode), AP, and HR elicited by intra-RVLM microinjection of 100, 200, or 300 pmol of the CB₁R agonist WIN55212-2 or its vehicle in conscious freely moving rats.

Fig. 3. A to C, time-course changes in Δ MAP (A), Δ HR (B), and RVLM-NO concentration (Δ NO; detected electrochemically in RVLM by a nafion-coated micro-electrode) (C) caused by intra-RVLM microinjection of 100, 200, or 300 pmol of the CB₁R agonist WIN55212-2 or its vehicle. Values are mean $\pm$ S.E.M. *, $P < 0.05$ versus vehicle. #, $P < 0.05$ versus 100 pmol. @, $P < 0.05$ versus 200 pmol. D, quantification of changes in integrated density of RVLM p-nNOS detected by Western blotting after unilateral intra-RVLM WIN55212-2 compared with the contralateral RVLM (control). Bands of p-nNOS and total nNOS were detected simultaneously from the same membrane by using a dual infrared fluorescence imager. p-nNOS integrated density was normalized to the corresponding total nNOS. Values are mean $\pm$ S.E.M. *, $P < 0.05$ versus the contralateral (control) RVLM.

TABLE 1

Baseline MAP and HR values before and after pretreatment with different drugs that were administered before intracisternal WIN55212-2 or its vehicle

Values are means $\pm$ S.E.M.

Treatment	n	MAP		HR	
		Before	After	Before	After
		mm Hg		beats/min	
Saline + vehicle	6	115.0 $\pm$ 6.0	113.3 $\pm$ 5.0	395 $\pm$ 12	400 $\pm$ 12
Saline + 15 μ g WIN55212-2	8	113.3 $\pm$ 5.0	115.0 $\pm$ 3.0	370 $\pm$ 10	368 $\pm$ 17
NPLA + 15 μ g WIN55212-2	8	111.3 $\pm$ 3.0	112.0 $\pm$ 3.0	375 $\pm$ 10	363 $\pm$ 13
NPLA + vehicle	5	113.0 $\pm$ 4.0	110.0 $\pm$ 4.0	398 $\pm$ 16	390 $\pm$ 20

scious rats because: 1) brainstem nNOS/NO signaling is critically implicated in the central regulation of blood pressure [although many studies have suggested a sympathoinhibitory/hypotensive effect for central NO, a few studies have implicated RVLM nNOS in sympathoexcitation (Martins-Pinge et al., 1999, 2007; Chan et al., 2003, 2004; Ally et al., 2007; Mayorov, 2007; Guo et al., 2009)]; and 2) many of the CB₁R-mediated neurobiological responses depend on nNOS/NO signaling (Makara et al., 2007; Jones et al., 2008; Carney et al., 2009). Here, we present the first evidence that early (5 min) activation (phosphorylation) of RVLM nNOS (Fig. 1) preceded the peak of central CB₁R-mediated pressor response (Fig. 4).

Nonetheless, as acknowledged in our recent studies (Ibrahim and Abdel-Rahman, 2011, 2012), the hemodynamic and neurochemical responses observed after intracisternal administration of WIN55212-2, involve complex neuronal inputs in addition to the RVLM. Therefore, in this study, we sought the first evidence that directly implicates localized RVLM nNOS-NO signaling in the central CB₁R-mediated

pressor response. We used a technique that permitted simultaneous measurement of blood pressure, heart rate, and real-time changes in NO (in vivo electrochemistry), which was used in our previous studies (Li and Abdel-Rahman, 2009). We showed that unilateral intra-RVLM microinjection of the CB₁R agonist WIN55212-2 (100, 200, or 300 pmol) elicited dose-dependent increases in blood pressure along with a slight increase in heart rate in conscious rats (Figs. 2 and 3). These BP and HR responses were not caused by a volume increase, because the vehicle, administered in the same volume, and repeated microinjections of lower doses of WIN55212-2 used in a preliminary study (see *Protocols and Experimental Groups*) had no effect on the measured variables. The slight increase in HR is contrary to the bradycardia induced by WIN55212-2 we and others have reported after its intracisternal administration (Niederhoffer and Szabo, 1999, 2000; Pfitzer et al., 2004; Ibrahim and Abdel-Rahman, 2011, 2012). The difference in the heart rate response might be caused by the localized effect of WIN55212-2 within the RVLM compared with the more widespread effect

Fig. 5. Changes in p-nNOS in rat RVLM elicited by intracisternal WIN55212-2 (15 μ g/rat) or an equal volume of its vehicle in animals pretreated with sterile saline, the CB₁R antagonist AM251 (30 μ g/rat i.c.), or the nNOS inhibitor NPLA (1 μ g/rat i.c.). Data are presented as integrated density ratio of p-nNOS to t-nNOS and expressed as percentage of control value (sterile saline + vehicle). Values are mean $\pm$ S.E.M. of four to five observations. *, $P < 0.05$ versus sterile saline + vehicle. #, $P < 0.05$ versus saline + 15 μ g of WIN55.

after intracisternal injection. It is noteworthy that our hemodynamic findings with intra-RVLM WIN55212-2 replicated the human response to marijuana/tetrahydrocannabinol smoking (Benowitz et al., 1979) and also agree with similar findings in anesthetized animals (Padley et al., 2003) despite the difference in the WIN55212-2 dose range used in the two studies. It is imperative to note that our electrochemical findings temporally linked the dose-related increase in local NO after intra-RVLM WIN55212-2 microinjection to the subsequent increases in BP. These findings along with the enhanced RVLM nNOS phosphorylation after intra-RVLM WIN55212-2 (Figs. 2 and 3) confirm the Western blot findings observed after intracisternal WIN55212-2 administration (Figs. 1 and 5). Together, these findings inferred a causal role for NO increase within the RVLM in the CB₁R-mediated pressor response.

Fig. 4. Representative tracings (left) and time-course changes (right) in Δ MAP and Δ HR evoked by intracisternal WIN55212-2 (WIN55; 15 μ g/rat) compared with equal volume of its vehicle in conscious rats pretreated, 30 min earlier, with the nNOS inhibitor NPLA (1 μ g/rat i.c.) or an equal volume of sterile saline. Values are mean $\pm$ S.E.M. of four to eight observations. *, $P < 0.05$ versus NPLA + vehicle. #, $P < 0.05$ versus NPLA + 15 μ g of WIN55.

A possible role for eNOS or iNOS-produced NO cannot be completely excluded and warrants additional studies. However, it should be remembered that nNOS-derived NO in the RVLM is implicated in pressor responses (Kishi et al., 2001; Chan et al., 2003; Ally et al., 2007; Martins-Pinge et al., 2007). Therefore, we used a selective nNOS inhibitor (NPLA) to further confirm this point in our model system. In support of our hypothesis, the pressor response evoked by intracisternal WIN55212-2 and the associated increases in p-nNOS level and the number of nNOS-ir neurons expressing c-Fos (denoting an increase in nNOS neuronal activity) in the RVLM all were attenuated by prior selective blockade of central CB₁R (AM251) or inhibition of nNOS phosphorylation (NPLA) (Figs. 4–6). Collectively, these findings clearly implicate these neurochemical responses in the central CB₁R-mediated pressor response.

Nonetheless, it is important to reconcile the present neurochemical findings with our previous findings, which implicated the enhancement of RVLM ERK1/2 signaling and inhibition of brainstem GABAergic neurotransmission in the central CB₁R-mediated pressor response in conscious rats (Ibrahim and Abdel-Rahman, 2011, 2012). First, nNOS-generated NO reduces GABA release in the dorsal periaqueductal gray matter and RVLM along with a pressor response (Chan et al., 2003; Karlsson et al., 2007; Martins-Pinge et al., 2007). Second, ERK1/2-dependent phosphorylation of nNOS in the RVLM mediates sympathoexcitation (Chan et al., 2004, 2005, 2010). Third, ERK1/2 negatively modulates GABA-A receptor signaling (Bell-Horner et al., 2006). Finally, we gathered important integrative and molecular data that linked WIN55212-2-evoked reduction in GABAergic activity (Ibrahim and Abdel-Rahman, 2011) and the concomitant increases in ERK1/2 (Ibrahim and Abdel-Rahman, 2012) and nNOS (this study) phosphorylation to the generated pressor response based on findings with the selective CB₁R antagonist AM251 in the same model system. This conclusion gains further credence because the molecular findings of our three studies were undertaken on RVLM tissues collected from the same animals that received AM251 or its vehicle before WIN55212-2. Indeed, the use of the intracisternal route permitted such studies because it enabled us to

Fig. 6. Confocal dual-channel images showing nNOS-ir neurons (green) and c-Fos immunoreactive (Fos-ir) cell nuclei (red) in RVLM of rats treated as described under *Materials and Methods* with intracisternal vehicle (A), 15 µg of WIN55212-2 (B), AM251 + 15 µg of WIN55212-2 (C), and NPLA + 15 µg of WIN55212-2 (D). White or yellow arrowheads indicate single labeled nNOS-ir neurons or c-Fos-ir cell nuclei, respectively. White arrows denote c-Fos/nNOS colabeled cells. Scale bar, 20 µm. Number of nNOS-ir neurons, c-Fos/nNOS dual-labeled neurons, and percentage of nNOS-ir neurons colocalized with c-Fos in the RVLM of control rats and treated rats is shown below. Bar graphs represent mean ± S.E.M. of data obtained from four to six coronal brainstem sections/animal (n = 3–5 rats/group) by using one-way ANOVA followed by Bonferroni comparison test. *, P < 0.05 compared with vehicle. #, P < 0.05 compared with WIN55212-2 alone.

perform the molecular studies on RVLM tissues collected from both sides of the brain. Nonetheless, future studies are warranted to investigate the interesting possibility that enhancement of RVLM p-nNOS/NO signaling constitutes the intermediate step between CB₁R-mediated elevation of ERK1/2 phosphorylation and GABAergic inhibition in the RVLM, which ultimately leads to sympathoexcitation/pressor response.

In summary, the present study highlights a pivotal role for RVLM p-nNOS-NO signaling in the central CB₁R-mediated pressor response in conscious rats because intra-RVLM WIN55212-2: 1) elicited parallel dose-dependent increases in RVLM NO and BP in conscious rats; 2) significantly increased RVLM nNOS phosphorylation; and 3) enhanced the activity (c-Fos) of RVLM nNOS-ir neurons and these neurochemical responses all were abrogated by prior blockade of central CB₁R (AM251) or selective inhibition of central nNOS (NPLA). The present findings are clinically relevant because they delineated, for the first time, a central molecular mechanism for the CB₁R-mediated pressor response in conscious unrestrained rats, which replicates the response in humans. Further studies are needed to elucidate the signaling pathway between ERK1/2 and nNOS phosphorylation and the inhibition of the GABA-A receptor signaling within the RVLM to elucidate the molecular mechanisms involved in the pressor response elicited by local CB₁R activation.

Acknowledgments

We thank Dr. Guichu Li and Kui Sun for technical assistance.

Authorship Contributions

Participated in research design: Ibrahim and Abdel-Rahman.

Conducted experiments: Ibrahim.

Performed data analysis: Ibrahim.

Wrote or contributed to the writing of the manuscript: Ibrahim and Abdel-Rahman.

References

- Ally A, Kabadi S, Phattanasarudee S, Patel M, and Maher TJ (2007) Neuronal nitric oxide synthase (nNOS) blockade within the ventrolateral medulla differentially modulates cardiovascular responses and nNOS expression during static skeletal muscle contraction. *Brain Res* 1150:21–31.
- Azad SC, Marsicano G, Eberlein I, Putzke J, Zieglgänsberger W, Spanagel R, and Lutz B (2001) Differential role of the nitric oxide pathway on Δ^9 -THC-induced central nervous system effects in the mouse. *Eur J Neurosci* 13:561–568.
- Bell-Horner CL, Dohi A, Nguyen Q, Dillon GH, and Singh M (2006) ERK/MAPK pathway regulates GABAA receptors. *J Neurobiol* 66:1467–1474.
- Bender TS and Abdel-Rahman AA (2010) Differential central NOS-NO signaling underlies clonidine exacerbation of ethanol-evoked behavioral impairment. *Alcohol Clin Exp Res* 34:555–566.
- Benowitz NL, Rosenberg J, Rogers W, Bachman J, and Jones RT (1979) Cardiovascular effects of intravenous Δ^9 -tetrahydrocannabinol: autonomic nervous mechanisms. *Clin Pharmacol Ther* 25:440–446.
- Carney ST, Lloyd ML, MacKinnon SE, Newton DC, Jones JD, Howlett AC, and Norford DC (2009) Cannabinoid regulation of nitric oxide synthase I (nNOS) in neuronal cells. *J Neuroimmune Pharmacol* 4:338–349.
- Chan JY, Chan SH, and Chang AY (2004) Differential contributions of NOS isoforms in the rostral ventrolateral medulla to cardiovascular responses associated with mevinphos intoxication in the rat. *Neuropharmacology* 46:1184–1194.
- Chan JY, Chan SH, Li FC, Tsai CY, Cheng HL, and Chang AY (2005) Phasic cardiovascular responses to mevinphos are mediated through differential activation of cGMP/PKG cascade and peroxynitrite via nitric oxide generated in the rat

- rostral ventrolateral medulla by NOS I and II isoforms. *Neuropharmacology* **48**: 161–172.
- Chan SH, Sun EY, and Chang AY (2010) Extracellular signal-regulated kinase 1/2 plays a pro-life role in experimental brain stem death via MAPK signal-interacting kinase at rostral ventrolateral medulla. *J Biomed Sci* **17**:17.
- Chan SH, Wang LL, and Chan JY (2003) Differential engagements of glutamate and GABA receptors in cardiovascular actions of endogenous nNOS or iNOS at rostral ventrolateral medulla of rats. *Br J Pharmacol* **138**:584–593.
- Friedemann MN, Robinson SW, and Gerhardt GA (1996) O-phenylenediamine-modified carbon fiber electrodes for the detection of nitric oxide. *Anal Chem* **68**:2621–2628.
- Guo ZL, Tjen-A-Looi SC, Fu LW, and Longhurst JC (2009) Nitric oxide in rostral ventrolateral medulla regulates cardiac-sympathetic reflexes: role of synthase isoforms. *Am J Physiol Heart Circ Physiol* **297**:H1478–H1486.
- Ibrahim BM and Abdel-Rahman AA (2011) Role of brainstem GABAergic signaling in central cannabinoid receptor evoked sympathoexcitation and pressor responses in conscious rats. *Brain Res* **1414**:1–9.
- Ibrahim BM and Abdel-Rahman AA (2012) Differential modulation of brainstem PI3K/Akt and ERK1/2 signaling underlies WIN55212-2 centrally mediated pressor response in conscious rats. *J Pharmacol Exp Ther* **340**:11–18.
- Institute of Laboratory Animal Resources (2010) *Guide for the Care and Use of Laboratory Animals*. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC.
- Jones JD, Carney ST, Vrana KE, Norford DC, and Howlett AC (2008) Cannabinoid receptor-mediated translocation of NO-sensitive guanylyl cyclase and production of cyclic GMP in neuronal cells. *Neuropharmacology* **54**:23–30.
- Karlsson GA, Chaitoff KA, Hossain S, Böhlke M, Maher TJ, and Ally A (2007) Modulation of cardiovascular responses and neurotransmission during peripheral nociception following nNOS antagonism within the periaqueductal gray. *Brain Res* **1143**:150–160.
- Kishi T, Hirooka Y, Sakai K, Shigematsu H, Shimokawa H, and Takeshita A (2001) Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release. *Hypertension* **38**:896–901.
- Li G and Abdel-Rahman AA (2007) Direct evidence for imidazoline (I₁) receptor modulation of ethanol action on norepinephrine-containing neurons in the rostral ventrolateral medulla in conscious spontaneously hypertensive rats. *Alcohol Clin Exp Res* **31**:684–693.
- Li G and Abdel-Rahman AA (2009) Estrogen-dependent enhancement of NO production in the nucleus tractus solitarius contributes to ethanol-induced hypotension in conscious female rats. *Alcohol Clin Exp Res* **33**:366–374.
- Li G, Wang X, and Abdel-Rahman AA (2005) Neuronal norepinephrine responses of the rostral ventrolateral medulla and nucleus tractus solitarius neurons distinguish the I₁ from the α 2-receptor-mediated hypotension in conscious SHR. *J Cardiovasc Pharmacol* **46**:52–62.
- Makara JK, Katona I, Nyíri G, Németh B, Ledent C, Watanabe M, de Vente J, Freund TF, and Hájos N (2007) Involvement of nitric oxide in depolarization-induced suppression of inhibition in hippocampal pyramidal cells during activation of cholinergic receptors. *J Neurosci* **27**:10211–10222.
- Martins-Pinge MC, Araújo GC, and Lopes OU (1999) Nitric oxide-dependent guanylyl cyclase participates in the glutamatergic neurotransmission within the rostral ventrolateral medulla of awake rats. *Hypertension* **34**:748–751.
- Martins-Pinge MC, Garcia MR, Zoccal DB, Crestani CC, and Pingue-Filho P (2007) Differential influence of iNOS and nNOS inhibitors on rostral ventrolateral medullary mediated cardiovascular control in conscious rats. *Auton Neurosci* **131**: 65–69.
- Mayorov DN (2007) Nitric oxide synthase inhibition in rostral ventrolateral medulla attenuates pressor response to psychological stress in rabbits. *Neurosci Lett* **424**: 89–93.
- Nassar N and Abdel-Rahman AA (2008) Brainstem phosphorylated extracellular signal-regulated kinase 1/2-nitric-oxide synthase signaling mediates the adenosine A_{2A}-dependent hypotensive action of clonidine in conscious aortic barodenervated rats. *J Pharmacol Exp Ther* **324**:79–85.
- Nassar NN, Li G, Strat AL, and Abdel-Rahman AA (2011) Enhanced hemeoxygenase activity in the rostral ventrolateral medulla mediates the exaggerated hemin-evoked hypotension in the SHR. *J Pharmacol Exp Ther* **339**:267–274.
- Niederhoffer N and Szabo B (1999) Effect of the cannabinoid receptor agonist WIN55212-2 on sympathetic cardiovascular regulation. *Br J Pharmacol* **126**:457–466.
- Niederhoffer N and Szabo B (2000) Cannabinoids cause central sympathoexcitation and bradycardia in rabbits. *J Pharmacol Exp Ther* **294**:707–713.
- Padley JR, Li Q, Pilowsky PM, and Goodchild AK (2003) Cannabinoid receptor activation in the rostral ventrolateral medulla oblongata evokes cardiorespiratory effects in anaesthetised rats. *Br J Pharmacol* **140**:384–394.
- Paxinos G and Watson C (2005) *The Rat Brain in Stereotaxic Coordinates*. Elsevier Academic Press, Boston.
- Pfister T, Niederhoffer N, and Szabo B (2004) Central effects of the cannabinoid receptor agonist WIN55212-2 on respiratory and cardiovascular regulation in anaesthetised rats. *Br J Pharmacol* **142**:943–952.
- Zhang J and Abdel-Rahman AA (2005) Mitogen-activated protein kinase phosphorylation in the rostral ventrolateral medulla plays a key role in imidazoline (I₁)-receptor-mediated hypotension. *J Pharmacol Exp Ther* **314**:945–952.

Address correspondence to: Dr. Abdel A. Abdel-Rahman, Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC 27834. E-mail: abdelrahmana@ecu.edu

---